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Respiratory Syncytial Virus Infection in BALB/c Mice Previously Immunized with Formalin-Inactivated Virus Induces Enhanced Pulmonary Inflammatory Response with a Predominant Th2-Like Cytokine Pattern

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Vaccination with formalin-inactivated respiratory syncytial virus (FI-RSV) caused excessive disease in infants upon subsequent natural infection with RSV. Recent studies with BALB/c mice have suggested that T cells are important contributors to lung immunopathology during RSV infection. In this study, we investigated vaccine-induced enhanced disease by immunizing BALB/c mice with live RSV intranasally or with FI-RSV intramuscularly. The mice were challenged with RSV 6 weeks later, and the pulmonary inflammatory response was studied by analyzing cells obtained by bronchoalveolar lavage 4 and 8 days after challenge. FI-RSV-immunized mice had an increased number of total cells, granulocytes, eosinophils, and $CD4^+$ cells but a decreased number of $CD8^+$ cells. The immunized mice also had a marked increase in the expression of mRNA for the Th2-type cytokines interleukin-5 (IL-5) and IL-13 as well as some increase in the expression of IL-10 (a Th2-type cytokine) mRNA and some decrease in the expression of IL-12 (a Th1-type cytokine) mRNA. The clear difference in the pulmonary inflammatory response to RSV between FI-RSV- and live-RSV-immunized mice suggests that this model can be used to evaluate the disease-enhancing potential of candidate RSV vaccines and better understand enhanced disease.

Respiratory syncytial virus (RSV) is the most important cause of serious respiratory disease in infants and young children. First infection frequently occurs in the presence of maternal antibodies, and reinfections are common throughout life. To date, efforts to develop a vaccine have failed. During the 1960s, infants vaccinated with a formalin-inactivated RSV (FI-RSV) vaccine experienced enhanced disease with subsequent natural infection (6, 13, 19, 20). The lack of understanding of the pathogenesis of this enhanced disease is still blocking clinical trials with subunit or inactivated RSV vaccines in naive children.

Viruses induce both antigen-specific antibody and T-cell responses, and the function of the latter has recently been emphasized as an important determinant in the inflammatory process. Cytokines, including proteins belonging to the interferon (IFN), interleukin (IL), and tumor necrosis factor families, are key to the phenotypic differentiation of T cells. In general, $CD4^+$ Th cells are subdivided into two types on the basis of cytokine production: Th1 clones, producing IFN-γ, IL-2, and TNF-β; and Th2 clones, producing IL-4, IL-5, IL-6, IL-10, and IL-13 (24, 30). These cytokines can inhibit each other's effects, regulate different aspects of the immune response, and produce various effects on inflammatory cells including macrophages, natural killer cells, and lymphocyte-activated killer cells. The impact of the memory T-cell response on the outcome of infection is demonstrated in a number of diseases including leishmaniasis and shistosomiasis, and in several of these, a Th2-like cytokine pattern has been associated with more severe disease and a Th1-like pattern has been associated with absence of disease (38).

In the past 10 years, considerable effort has been directed toward characterizing enhanced disease that occurs after vaccination with FI-RSV. Studies of mice have identified differences between FI-RSV vaccination and live RSV infection, suggesting that the subtype of T cell induced by FI-RSV may be important to the pathogenesis of enhanced disease. Mice immunized with FI-RSV show, even in the absence of disease symptoms, increased histopathologic changes in the lungs after RSV challenge. This enhanced histopathology can be abrogated by depletion of $CD4^+$ T cells (8) or both of the cytokines IL-4 and IL-10 (7). Finally, mice immunized with FI-RSV have a high ratio of IL-4 to IFN-γ mRNA in their lungs after challenge, suggestive of a Th2-like response, whereas mice immunized with live RSV have a low ratio of IL-4 to IFN-γ mRNA in their lungs, suggestive of a Th1-like response (14).

The aim of the present study was to characterize the cytokine patterns and the pulmonary inflammatory response to RSV in BALB/c mice primed by prior immunization with vaccine or live virus. We chose to investigate the cells in bronchoalveolar lavage (BAL) fluid, and the result of this approach showed clear differences between different immunizations in the cell numbers, types of cells, and cytokine mRNA expression.

MATERIALS AND METHODS

Design. BALB/c mice were immunized with FI-RSV intramuscularly (i.m.) or live RSV intranasally (i.n.), challenged 6 weeks later with live virus, and killed 4 or 8 days after challenge. Blood, BAL fluid, and lungs were harvested to measure serum antibody responses and determine the number and characteristics of inflammatory cells, including their cytokine mRNA profiles, and viral titers in the lungs.

Virus and immunization antigens. RSV A2 was cloned by limiting dilution three times in HEp-2 cells in 96-well microtiter plates. Stock virus was propa-

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TABLE 1. Primers and probes used in mouse cytokine mRNA cDNA detection by semiquantitative PCR

Cytokine	Primer sequence	Probe sequence	Reference
IFN-γ	(+) 5′-AACGCTACACACTGCATCT-3′ (−) 5′-GAGCTCATTG_AATGCTTGG-3′	(−) 5′-TCGCCTTGCTGTTGCTGT-3′	23[a]
IL-2	(+) 5′-AACAGCGCACCCACTTCAA-3′ (−) 5′-TTGAGATGATGCTTTGACA-3′	(−) 5′-GTTCATCTTCTAGGCACTG-3′	23[a]
IL-4	(+) 5′-TAGTTGTCATCCTGCTCTT-3′ (−) 5′-CTACGAGTAATCCATTTGC-3′	(−) 5′-GATGATCTCTCTAAGTG-3′	23[a]
IL-5	(+) 5′-AAGGATGCTTCTGCACTTGA-3′ (−) 5′-ACACCAAGGAACTCTTGCA-3′	(−) 5′-TCCGTCTCTCCTCGCCAC-3′	23[a]
IL-10	(+) 5′-CAGCCGGGAAGACAATAAC-3′ (−) 5′-TCCCTGGATCAGATTTAGAGA-3′	(−) 5′-GGCATCACTTCTACCAGG-3′	21[b]
IL-12	(+) 5′-GACACGCCTGAAGAAGATGAC-3′ (−) 5′-CGCCATTCCACATGTCACTGC-3′	(−) 5′-TGGAGCAGCAGATGTGAG-3′	33[b]
IL-13	(+) 5′-TCTCCCCAGCAAAGTCTGAT-3′ (−) 5′-CTGGATTCCCTGACCAACAT-3′	(−) 5′-GGTCTCCCAGCCTCCCCGATA-3′	4[b]
β-Actin	(+) 5′-ATGGATGACGATATCGCT-3′ (−) 5′-ATGAGGTAGTCTGTCAGGT-3′	ND[c]	23[a]

[a] Reference for primer and probe sequences; underlined nucleotides modified.
[b] Reference for gene sequence used to design primers and probe.
[c] ND, control for RNA extraction, cDNA preparation, and PCR; the product was analyzed by agarose gel electrophoresis and ethidium bromide staining only.

gated in HEp-2 or Vero cells maintained in 5% fetal calf serum–Eagle minimal essential medium supplemented with 2 mM glutamine, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 100 U of penicillin G per ml, and 100 μg of streptomycin per ml (5% FCS–MEM). Nearly confluent monolayers in 150-cm^2 flasks were inoculated with 2.5×10^6 PFU of virus (~0.05 PFU per cell) in 5 ml of 5% FCS–MEM per flask by rocking at 3 rpm for 1 h at room temperature. Medium was then added to a volume of 30 ml per flask, and the cultures were rocked at 36.5°C. When early cytopathic effect was observed, the medium was replaced with 25 ml of serum-free MEM per flask and the cultures were incubated without rocking in a CO_2 incubator at 36.5°C. At full cytopathic effect, the cells were suspended into the medium, sonicated, and centrifuged at $1,000 \times g$ for 10 min at 4°C.

FI-RSV was prepared by the method used for the original vaccine tested in the 1960s (20). Clarified cell culture supernatant (2×10^6 PFU/ml) was incubated for 3 days with formalin (1:4,000) at 37°C and centrifuged at 4°C for 1 h at $50,000 \times g$. The resulting pellet was resuspended 1:25 in serum-free MEM and precipitated with aluminum hydroxide (4 mg/ml) for 30 min. The precipitate was collected by centrifugation for 30 min at $1,000 \times g$, resuspended 1:4 in serum-free MEM, and stored at 4°C. The working dilution of the vaccine was made in phosphate-buffered saline immediately before use. RSV for live-virus immunizations (10^7 PFU/ml) was concentrated from the clarified cell culture supernatant by centrifugation at 4°C for 1 h at $50,000 \times g$, resuspended in serum-free MEM, and stored at −70°C. The challenge virus (10^8 PFU/ml) was partially purified from clarified cell culture supernatant by ultracentrifugation (twice) onto a cushion of 1.15 M sucrose in 1 M $MgSO_4$–0.15 M NaCl–50 mM HEPES (12) and stored at −70°C. The working dilutions of live-virus preparations were made in serum-free MEM immediately before use. Control preparations were made in identical fashion, with uninfected HEp-2 cells or parainfluenza virus type 3 (PIV3)-infected Vero cells.

Mice, immunizations, challenge, and specimen collection. Female BALB/c mice, 8 weeks old, were obtained from Harlan Sprague-Dawley, Indianapolis, Ind. They were maintained under specific-pathogen-free conditions and used when 8 to 16 weeks old. Before i.n. intranasal inoculation, mice were anesthetized with ketamine and xylazine and inoculated as described by Graham et al. (15). The animals were immunized on day 0 with 0.05 ml of FI-RSV i.m. or with 50 or 100 μl of live virus i.n. and challenged with live virus 6 weeks later. At 4 or 8 days after challenge, each mouse was anesthetized with an intraperitoneal injection of 0.5 ml of Avertin (20 mg of 2,2,2-tribromoethanol per ml and 20 μl of *tert*-amyl alcohol per ml in distilled water), the diaphragm was cut, and the animal was exsanguinated by heart puncture. BAL was performed through a catheter inserted into the trachea and by flushing the lungs with 1 ml of 12 mM lidocaine in phosphate-buffered saline six times in and out (27). Immediately after the lavage, 0.5 ml of BAL fluid was mixed with 1 ml of RNAzol B (Biotecx Laboratories, Houston, Tex.) in a 2-ml polypropylene tube (Marsh BioMedical Products, Rochester, N.Y.), quick-frozen in an ethanol–dry-ice bath, and stored at −70°C. The rest of the fluid was kept cold for cytospin preparations. The lungs were removed, excess blood was blotted onto a sterile gauze pad, and the lungs were placed in a 2-ml screw cap conical tube (Sarstedt, Newton, N.C.) containing 1 ml of 10% FCS–MEM, quick-frozen in an ethanol–dry-ice bath, and stored at −70°C. For flow cytometry experiments, BAL was done a second time with fresh solution, and the fluid was combined with the first fluid minus 0.5 ml used for RNA extraction and 0.1 ml used for virus titer determination.

Virus infectivity assay. The lungs were individually homogenized in their collection tubes. With the lungs still frozen, the tube was filled with sterile 1-mm-diameter glass beads (Biospec Products, Bartesville, Okla.) and 10% FCS–MEM and the tissue was disrupted in a Mini-BeadBeater homogenizer (Biospec Products) by vibrating at 5,000 rpm for 60 plus 20 s, with intermittent cooling in an ice bath. The suspension was centrifuged for 1 min at $10,000 \times g$, and the supernatant (1.2 ml) on top of the beads was removed and increased to a volume of 2 ml with 10% FCS–MEM and then centrifuged as above. The resulting supernatant was regarded as 6% (wt/vol) lung homogenate. Tenfold dilutions of virus preparations or fivefold dilutions of lung homogenates in 200-μl volumes were added in duplicate to 24-well plates with 80 to 100% confluent HEp-2 cells under 0.8 ml of fresh 5% FCS–MEM. The plate was centrifuged for 45 min at $1,000 \times g$ at 27°C and incubated for 18 h in a CO_2 incubator at 36.5°C. Infected cells were detected by immunoperoxidase staining as described previously (41), using a mixture of monoclonal antibodies (131-4g, 133-1h, and 92-11c [2]) that had been purified by protein G-Sepharose (Pharmacia, Piscataway, N.J.) chromatography and conjugated to preactivated horseradish peroxidase (Pierce, Rockford, Ill.) as specified by the manufacturer. Infected cells were counted with an inverted microscope.

Antibody assays. After the blood obtained by heart puncture was allowed to clot in a dry test tube and centrifuged at $1,000 \times g$ for 10 min, the serum was collected and stored below −20°C. RSV-specific immunoglobulin G (IgG) antibodies were measured by enzyme-linked immunosorbent assay (43) modified for RSV. Briefly, microtiter plates were coated with RSV-infected or uninfected cell lysate antigen prepared as described previously (11). Fourfold serum dilutions starting at a 1:100 dilution were incubated in the antigen-coated wells, and bound antibodies were detected with goat anti-mouse IgG (heavy plus light chains) labelled with horseradish peroxidase. Anti-RSV IgG titer was expressed as the highest dilution giving a specific absorbance of ≥0.05. Neutralizing antibodies were measured without complement in fourfold serum dilutions, starting at a 1:10 dilution, by a microneutralization test (2).

BAL cell stainings and flow cytometry analysis. Freshly collected BAL cells were cytocentrifuged onto microscopic slides and fixed and stained with Wright's reagent (Accustain; Sigma, St. Louis, Mo.). A 20-μl portion of unprocessed cells was stained with crystal violet, and the total number of cells was estimated microscopically by using a Reichert-Jung hemacytometer. The number of macrophages and eosinophils in BAL specimens was determined in 10 100× power fields by oil immersion light microscopy.

$CD4^+$ and $CD8^+$ T-cell populations were determined by flow cytometry. BAL specimens were washed with isotonic saline and divided into four aliquots, and

each was resuspended in 50 μl of fluorescence-activated cell sorter (FACS) wash buffer (2% inactivated FCS and 0.1% NaN_3 in phosphate-buffered saline [pH 7.2]). Optimal dilutions of fluorescein isothiocyanate-conjugated goat antibodies to mouse CD4, phycoerythrin-conjugated goat antibodies to mouse CD8, or respective isotype controls (PharMingen, San Diego, Calif.) in 2.5-μl volumes were added to each tube, and the mixtures were incubated at 4°C for 30 min in the dark. The cells were washed once with FACS wash buffer and fixed with 0.5 ml of 1% formaldehyde in isotonic saline. They were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.) by collecting 10,000 events per run and using the forward-side-scatter plot to define the lymphocyte population for counting the $CD4^+/CD8^+$ cells. The BAL method may slightly overestimate the number of $CD8^+$ cells, because they are more easily washed out from the lungs (22).

RNA extraction and semiquantitative analysis. Oligonucleotide primers were synthesized and biotinylated as described elsewhere (26), and probes were labelled with ruthenium chelate (Perkin-Elmer, Branchburg, N.J.) as specified by the manufacturer. The BAL specimen in RNAzol B was thawed and subjected to RNA extraction and reverse transcriptase reaction with poly(dT) primers, as described elsewhere (3). The cDNA was amplified by PCR with primers specific to mouse IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, and β-actin (Table 1). Each PCR was performed in a 50-μl volume, with final concentrations of 50 mM Tris-HCl (pH 9.0), 3 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 5 mM dithiotreitol, 10 to 35 pmol of primers, 2.5 U of *Taq* polymerase, 0.2 mM deoxynucleoside triphosphates, and 5 μl (1/16 of total cDNA) of template. The actual primer concentration used was optimized for each lot of each cytokine primer. The first denaturation was performed at 94°C for 5 min. The amplification cycles were 94°C for 75 s, 60°C (53°C for IL-10 and IL-12) for 75 s, and 72°C for 75 s. The last extension was 5 min at 72°C. The cytokine and β-actin cDNAs were amplified for 35 and 22 cycles, respectively. In some experiments, IFN-γ and IL-13 PCRs were also run for 31 cycles. cDNA from concavalin A- or (for IL-12) RSV-stimulated mouse splenocytes was used as positive control and water was used as negative control in each PCR. PCR products were electrophoresed on agarose gels and stained with ethidium bromide. A positive band for β-actin was used as the indicator of appropriate cDNA preparation.

For semiquantitative detection of cytokine mRNAs, each PCR product was hybridized with 10 to 20 pmol of ruthenium chelate-labelled probe complementary to an internal sequence of the amplified plus strand extended from the biotinylated plus primer. The biotinylated primer was captured with 2.8 μm of streptavidin-coated magnetic beads (Dynal, Great Neck, N.Y.), and the amount of hybridized probe was quantitated by measurement of electrochemiluminescence generated from ruthenium chelate, using Perkin-Elmer QPCR reagents and the Perkin-Elmer QPCR System 5000 analyzer. Results were expressed as LUM units (sample luminosity value − negative control luminosity value). A BAL specimen was considered positive for the respective cytokine mRNA if the luminosity value of the specimen was greater than twice that of the negative control.

RESULTS

Viral replication and antibody response. Viral titers in the lungs were determined 4 days after challenge, the time when virus titers peak in mice (15, 37). In our hands, prior BAL caused an approximately 40% decrease in viral titer in lung homogenate. Practically no infectious virus was detectable in the lungs of mice immunized with live RSV, whereas the lungs of FI-RSV-immunized mice had an approximately 50-fold mean reduction in virus titers (Table 2). No correlations were found between viral replication as indicated by titer in the lungs and the number or type of BAL cells within a treatment group.

In early experiments, antibody titers prior to challenge were almost identical to the titers obtained 4 days after challenge (data not shown). Antibody measurements from sera collected at harvest of the animals were thus used to measure the response to vaccination. Individual differences in antibody titers correlated with viral titers in the lungs but not with other parameters studied.

Although we attempted to use doses of live RSV or FI-RSV that induced similar levels of RSV antibodies, the doses we chose resulted in higher levels of antibody and protection for live RSV than for FI-RSV (Table 2). Doses of FI-RSV between 0.2×10^5 and 5×10^5 PFU equivalents gave similar patterns of cytokine mRNA (data not shown).

Analyses of the cells in the BAL fluid. The number of cells in BAL fluid after RSV challenge was larger in immunized mice than in mock-immunized or nonimmunized mice. The largest cell numbers were observed with FI-RSV 8 days after challenge, suggesting an enhanced inflammatory response (Table 3). Untreated mice or immunized mice that were mock challenged had 0.5×10^5 to 1.5×10^5 cells per ml BAL fluid at either time point. Figure 1 illustrates representative flow cytometry results with BAL cells from two mice, one immunized with live RSV and one immunized with FI-RSV, harvested 8 days after challenge with live virus. Mice immunized with FI-RSV had an increased number of cells with elevated side scatter, a measure of cell granularity, and a decreased number of cells with CD8 surface markers.

Table 3 summarizes the flow cytometry data for the different treatment groups. On day 4, all mice had more than twice as many $CD4^+$ cells as $CD8^+$ cells, the ratio being highest among FI-RSV recipients. On day 8, the $CD4^+/CD8^+$ ratio was ≤1 in

TABLE 2. Anti-RSV antibody response in serum and RSV replication in the lungs of RSV-infected BALB/c mice[a]

Expt	Immunization antigen[b] and route	Vol (μl)	Dose (PFU)[c]	No. of mice	IgG titer (mean $\log_2$ ± SD)	NA titer (mean $\log_2$ ± SD)	Virus titer (mean $\log_{10}$ PFU/g ± SD)
1	RSV i.n.	100	10^6	6	12.6 ± 1.1	8.8 ± 0.5	<1.6[d]
	FI-RSV i.m.	50	10^5	6	9.0 ± 1.2	5.2 ± 1.0	2.8 ± 1.2[d]
	FI-HEp-2 i.m.	50	0	3	<6.6	<3.3	4.3 ± 0.2
	HEp-2 i.n.	100	0	3	<6.6	<3.3	4.1 ± 0.3
2	RSV i.n.	100	10^5	4	12.6 ± 0.0[d]	ND[e]	<1.6
	FI-RSV i.m.	50	10^5	4	10.6 ± 0.8	ND	2.2 ± 0.9
	FI-HEp-2 i.m.	50	0	4	<6.6	ND	4.1 ± 0.2
	HEp-2 i.n.	100	0	4	<6.6	ND	4.0 ± 0.2
3	RSV i.n.	50	10^5	5	10.6 ± 0.7	6.9 ± 1.3	1.6 ± 0.2
	FI-RSV i.m.	50	10^5	4	8.9 ± 1.7	4.3 ± 1.4	2.3 ± 1.0
	RSV^{Vero} i.n.	50	10^5	5	11.6 ± 0.0	6.7 ± 0.5	<1.6
	$FI\text{-}RSV^{Vero}$ i.m.	50	10^5	5	9.8 ± 1.6	4.5 ± 1.5	2.2 ± 0.9
	$FI\text{-}PIV3^{Vero}$ i.m.	50	0	5	<6.6	<3.3	4.6 ± 0.1
	$PIV3^{Vero}$ i.n.	50	0	5	<6.6	<3.3	4.4 ± 0.3

[a] Mice were immunized as indicated, challenged 6 weeks later with 10^6 PFU of live RSV in a 100-μl inoculum, and harvested 4 days after challenge.
[b] RSV and FI-RSV were produced in the HEp-2 cell line if not otherwise indicated.
[c] PFU of live RSV in live virus inoculum or in FI-RSV before inactivation. Uninfected and PIV3-infected cell controls were diluted like their respective RSV antigen preparations.
[d] $n = 3$.
[e] ND, not done.

TABLE 3. Characteristics of the cell populations in the BAL fluid of RSV-infected BALB/c mice[a]

Immunization antigen and route	Day	Total cell no. (10^5)/ml	Granular cells[b] (%)	Lymphocytes			Eosinophil/macrophage ratio[c]
				% of $CD4^+$	% of $CD8^+$	$CD4^+/CD8^+$ ratio	
RSV i.n.	4	2.6 ± 0.4	2.6	17.0	6.9	2.5	0.0 ± 0.0[d]
	8	7.3 ± 0.6	6.5 ± 1.1[d]	11.1 ± 0.7	29.2 ± 2.6[d]	0.4 ± 0.1[d]	0.0 ± 0.0[d]
FI-RSV i.m.	4	3.9 ± 1.5	11.6	18.2	4.1	4.4	0.6 ± 0.3
	8	14.9 ± 5.2	22.7 ± 4.9	14.9 ± 4.5	5.5 ± 4.7	3.6 ± 1.6	1.1 ± 0.3
FI-HEp-2 i.m.	4	1.6 ± 0.6[d]	2.0	11.3	4.1	2.8	0.1 ± 0.1[d]
	8	2.7 ± 0.5[d]	8.0 ± 2.7[d]	11.7 ± 1.4	17.6 ± 4.6[d]	0.7 ± 0.2[d]	0.3 ± 0.4
HEp-2 i.n.	4	1.4 ± 0.7[d]	8.4	16.1	6.5	2.5	0.0 ± 0.0[d]
	8	3.5 ± 0.2[d]	8.7 ± 4.9[d]	9.1 ± 0.6	11.7 ± 3.2	0.8 ± 0.2[d]	0.0 ± 0.1[d]

[a] Mice were immunized with 10^5 PFU of RSV or equivalent dilution of the indicated antigen, challenged 6 weeks later with 10^6 PFU of live virus, and harvested 4 or 8 days after challenge. Results are mean ± SD for three or four mice or pooled BAL specimens from four mice.
[b] Cell population with an average forward-scatter/side-scatter ratio of 1.0 to 1.25 by flow cytometry analysis.
[c] Result of Wright staining of cytospin BAL cells in a separate experiment. Macrophages and eosinophils were counted in 10 100× power fields. Average macrophage counts were 121 ± 45 (day 4) and 93 ± 30 (day 8), without marked differences between immunization groups.
[d] Significant difference ($P < 0.05$ by Mann-Whitney U test) compared with FI-RSV-immunized animals.

all mice except those immunized with FI-RSV. This difference in ratio corresponded to a lower percentage of $CD8^+$ lymphocytes and a higher percentage of $CD4^+$ lymphocytes (Table 3). After accounting for the total number of BAL cells, FI-RSV-immunized mice had on average less than half the number of $CD8^+$ cells and more than twice the number of $CD4^+$ cells as did live-virus-immunized mice. The percentage of double-positive T cells was less than 3%, with no apparent differences between the treatment groups.

Light microscopy showed another difference between FI-RSV-immunized mice challenged with RSV and all the other groups: abundance of eosinophils. Since macrophages were easier to identify than other cell types and the number present in BAL fluid specimens was fairly consistent between treatment groups, we standardized the number of eosinophils to the number of macrophages (Table 3). Macrophages represented about 20% of BAL cells from immunized mice and 40% of BAL cells from mock-immunized mice. Only one mouse, immunized with FI–HEp-2, had a percentage of eosinophils comparable to that observed in the FI-RSV-immunized mice. The eosinophils had characteristic pink staining of the granules but were often hyperlobulated, a typical finding in eosinophilia (34). We expect that eosinophils were a major cell type in the granular cell population seen by flow cytometry.

Cytokine pattern. The QPCR system provided a specific and reproducible way to estimate relative levels of PCR products. Luminosity values for negative controls were between 30 and 150, and within any PCR run, the standard deviation (SD) of eight negative controls was always less than 30% of the mean of the negative controls. At 35 (31 for IFN-γ and IL-13) cycles, twofold dilutions of the positive-control cDNA gave a quantitative decrease in LUM units out to a 16- to 64-fold dilution. A fourfold dilution of the template corresponded to a ca. 50% decrease in LUM units.

We first studied mRNA levels for IFN-γ, IL-2, IL-4, and IL-5 by using 35 PCR cycles (Fig. 2A). RSV challenge induced a strong expression of IFN-γ mRNA in the BAL cells of all mice but relatively lower expression on day 4 in mock-immunized animals. This difference was more pronounced after 31 amplification cycles (data not shown). IL-2 and IL-4 mRNA levels appeared to be higher in mice immunized with either live RSV or FI-RSV compared with mock-immunized mice on day 4, and levels of both cytokines appeared higher in the FI-RSV group than in all other groups on day 8 (Fig. 2A). IL-2 mRNA levels in mice immunized with live RSV, FI-RSV, and any control were above cutoff in 29, 20, and 0% of specimens on day 4 and in 20, 80, and 23% of specimens on day 8, respectively. IL-4 mRNA levels in mice immunized with live RSV, FI-RSV, and any control were above cutoff in 71, 87, and 13% of specimens on day 4 and in 0, 67, and 18% of specimens on day 8, respectively. Levels of IL-5 mRNA were clearly higher

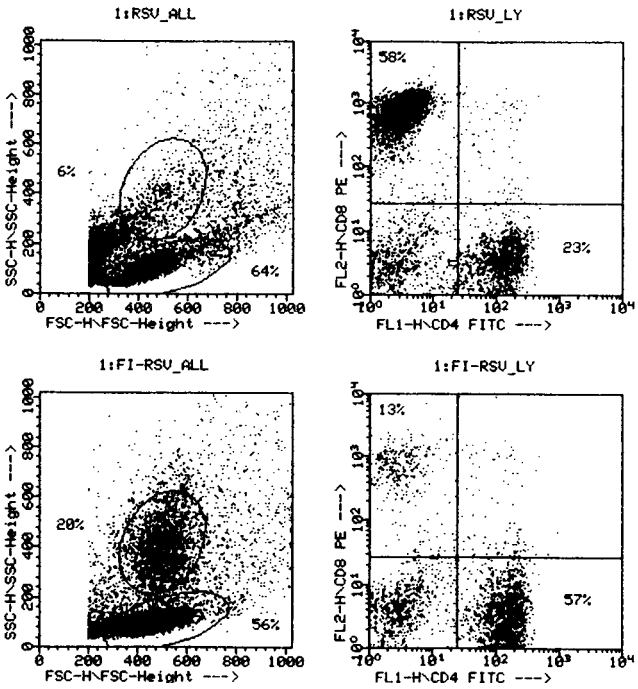

FIG. 1. Flow cytometry analysis of BAL cells from RSV-infected BALB/c mice. Mice were immunized i.n. with live RSV or i.m. with FI-RSV, challenged with live virus 6 weeks later, and harvested 8 days after challenge. RSV_ALL and FI-RSV_ALL indicate forward-scatter/side-scatter dot plots of all cells in a single BAL specimen. Outlined areas indicate the granular-cell populations (upper areas) and lymphocyte populations (lower areas). RSV_LY and FI-RSV_LY indicate CD4/CD8-specific fluorescence dot plots of the respective lymphocyte populations.

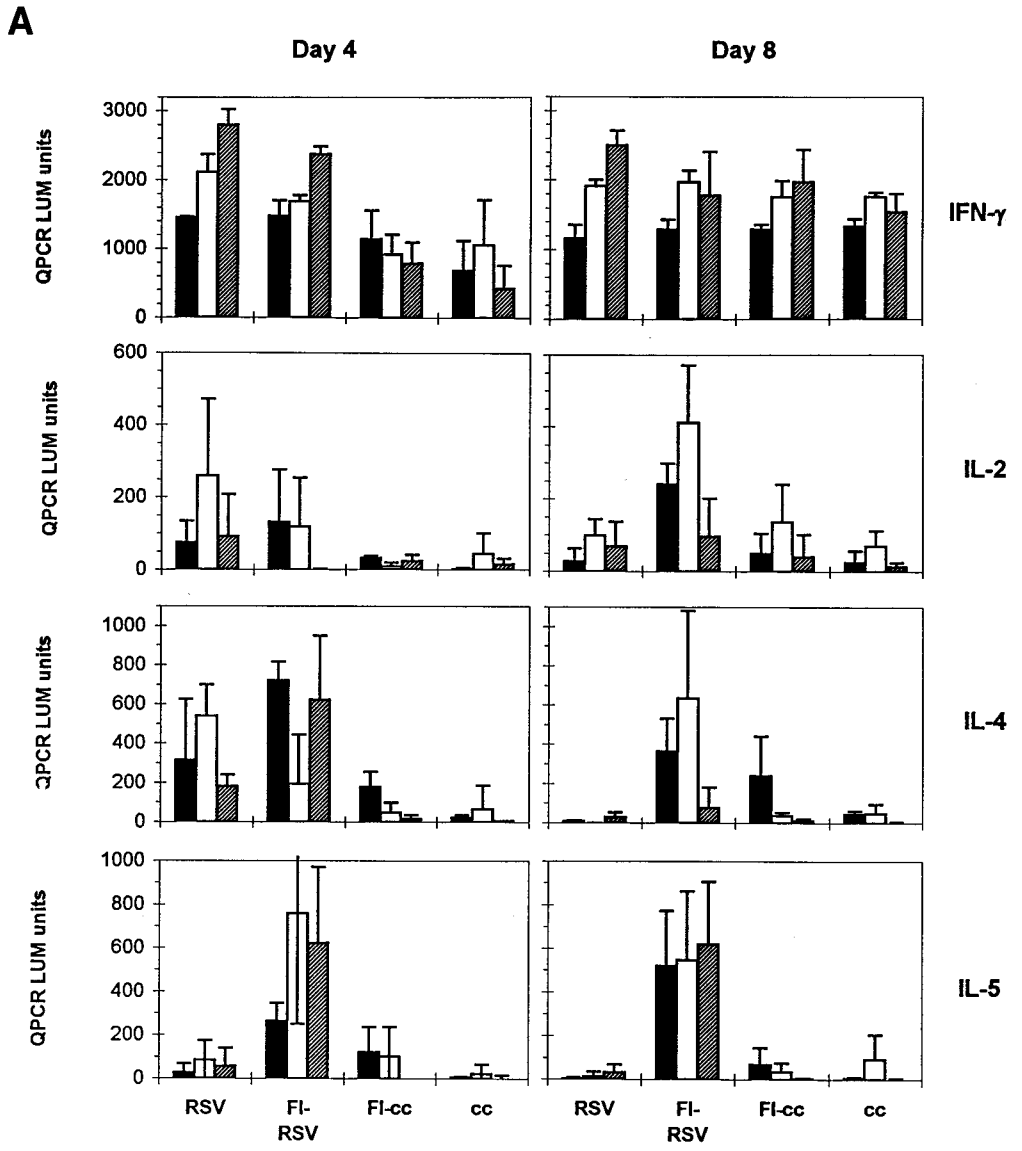

FIG. 2. Expression of IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-12, and IL-13 mRNAs in BAL cells from RSV-infected BALB/c mice. Mice were immunized i.n. with live RSV, i.m. with FI-RSV, or with respective cell controls (cc), challenged with live virus 6 weeks later, and harvested 4 or 8 days after challenge, and relative levels of IFN-γ, IL-2, IL-4, and IL-5 (A) and IL-10, IL-12, and IL-13 (B) mRNAs were determined. Immunizing antigens were 10^6 PFU of live RSV 10^5 PFU equivalents of FI-RSV, or respective uninfected HEp-2 cell controls (experiment 1 in Table 2) (solid bars); 10^5 PFU of live RSV, 10^5 PFU equivalents of FI-RSV, or respective uninfected HEp-2 cell controls (experiment 2 in Table 2), (open bars); or 10^5 PFU of live RSV, 10^5 PFU equivalents of FI-RSV, or respective PIV3-infected Vero cell controls (experiment 3 in Table 2 showing data for antigens produced in Vero cells only) (hatched bars). Results are expressed as mean specific luminosity value and SD of PCR determinations with three to five mice. The difference in mRNA levels between live-RSV- and FI-RSV-immunized mice was significant ($P < 0.05$ by the Mann-Whitney U test) for IL-2 and IL-4 in two of three experiments on day 8, for IL-5 in three of three experiments on days 4 and 8, for IL-10 in two of two experiments on day 8, for IL-12 in one of two experiments on day 4, and for IL-13 in one of two and two of two experiments on days 4 and 8, respectively.

in the FI-RSV-immunized group than in all other groups both 4 and 8 days after challenge. Furthermore, there was a positive correlation between the signal for IL-5 mRNA and the eosinophil/macrophage ratio ($r = 0.77$ by linear regression analysis). Eosinophilia was apparent when the IL-5 mRNA luminosity value of the same specimen was more than three times the negative control value.

We also examined the cDNA from two sets of experiments for IL-10, IL-12, and IL-13 mRNA with 35-cycle PCR assays (Fig. 2B). BAL cells from live-RSV- or FI-RSV-immunized mice consistently expressed IL-10 mRNA on day 4. On day 8, IL-10 mRNA was found in specimens from all mice but the levels were relatively lower in mice immunized with live virus. Expression of IL-12 mRNA on day 4 was strikingly lower in FI-RSV-immunized mice than in other groups. IL-13 mRNA levels were elevated in FI-RSV-immunized mice both at 4 and 8 days after challenge. When a 31-cycle PCR assay was used, only FI-RSV-immunized mice were positive for IL-13 mRNA (data not shown).

Differences in the titer and volume of live RSV inoculum used to immunize mice (Table 2) did not affect the pattern of cytokine mRNA (Fig. 1 and 2). Results were also not affected by use of Vero instead of HEp-2 cells to grow virus for live-RSV and FI-RSV immunizations (Fig. 1 and data not shown).

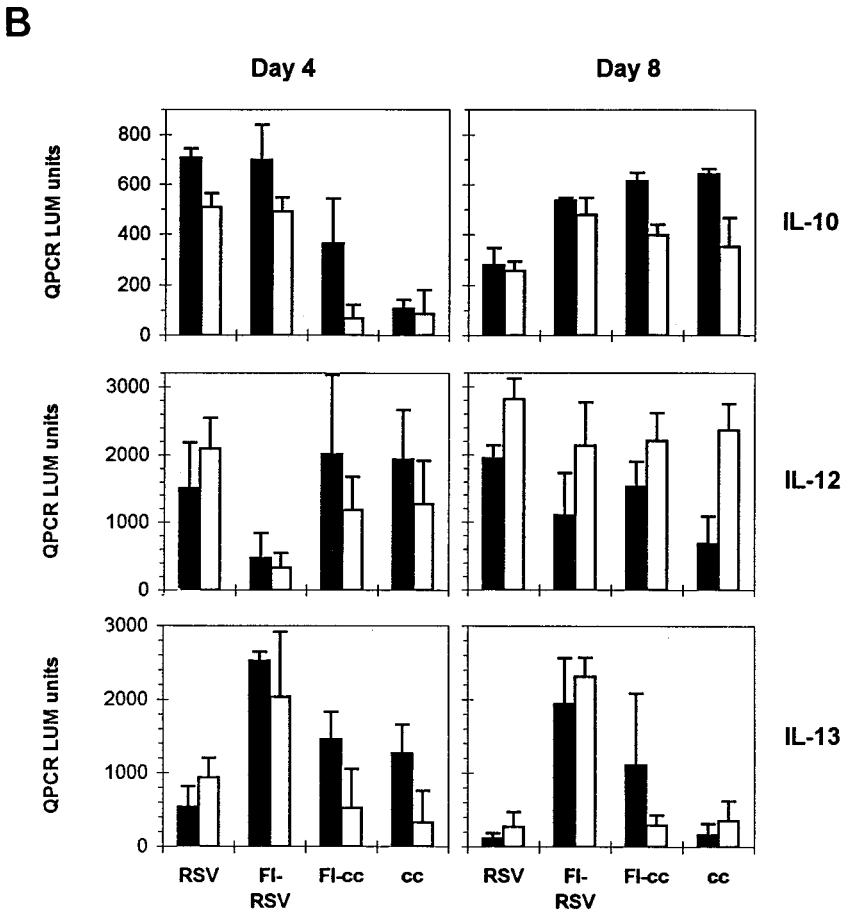

FIG. 2—*Continued.*

Finally, the pattern of cytokine mRNA from mice immunized with PIV3 or FI-PIV3 (Fig. 2A, experiment 3) was similar to that from mice immunized with other control antigens. BAL cells from unchallenged mice or mice challenged with mock-infected cell culture material had no or very low levels of all cytokine mRNAs.

DISCUSSION

In the present report, we describe assays that provide clear measures of enhanced pulmonary inflammatory response to RSV in BALB/c mice immunized with FI-RSV. In addition, we demonstrate that BAL cells involved in this response express several cytokine mRNAs of a Th2-like repertoire. Previous studies with cotton rats or mice have focused on lung histopathology based on numbers of inflammatory cells surrounding the alveoli and bronchioles (7, 8, 18, 25, 32). These studies are labor-intensive and subjective and have given inconsistent results between laboratories (18, 25). In contrast, we found the number and type of BAL cells in differently immunized mice to be markedly dissimilar in response to RSV challenge. The number of cells in the BAL fluid was moderately informative (Table 3). When we compared results obtained with challenged mice immunized with live virus with results obtained with those immunized with FI-RSV, we observed no significant difference in BAL specimens collected 4 days after challenge but found that the number of cells in the FI-RSV-immunized group was much larger 8 days after challenge. The minimal difference in the cell numbers on day 4 may explain some of the variation observed in earlier histopathologic studies in which day 4 was the only end point (7, 8). The type of BAL cells was more informative. The total number of T lymphocytes infiltrating the lungs and recovered by BAL was similar in FI-RSV- and live-RSV-immunized mice, but the ratio of the $CD4^+$ to $CD8^+$ lymphocytes was different between the groups, especially 8 days postchallenge (Table 3). The finding of a high ratio of $CD4^+$ to $CD8^+$ T cells with augmented cellular infiltration associated with FI-RSV vaccination is consistent with the hypothesis that $CD4^+$ cells and not $CD8^+$ cells are involved in enhanced histopathology in FI-RSV-immunized mice, as suggested by T-cell-subset depletion studies (8).

Flow cytometry analysis demonstrated another difference between live-RSV and FI-RSV immunization, i.e., in the number of granular cells after RSV challenge. There was an approximately sevenfold increase in the total number of granular BAL cells in FI-RSV recipients compared with live-RSV recipients both 4 and 8 days after challenge. We are in the process of further characterizing these cells, but we suspect that they are largely eosinophils on the basis of light-microscopic studies of BAL cells from comparable immunized mice. The demonstration of pulmonary eosinophilia in mice challenged with RSV after i.m. FI-RSV immunization is of special interest, since the lungs of two children who were vaccinated with FI-RSV and succumbed to natural infection also contained excess eosinophils (20). In one study, children vacci-

nated with FI-RSV had a peripheral eosinophilia after natural RSV infection (6). Openshaw et al. (29) described pulmonary eosinophilia after RSV challenge in mice immunized with RSV G (but not F, N, or SH) protein in a recombinant vaccinia virus vector. They also found eosinophils in BAL specimens from challenged mice that had been immunized intradermally with whole RSV in incomplete Freund's adjuvant. In addition, adoptive transfer of the G (but not F or M2) protein-specific T cells induced pulmonary eosinophilia in RSV-infected mice (1). Identifying the trigger for the eosinophilia should help clarify the pathogenesis of the enhanced inflammation in the FI-RSV-immunized mice.

In agreement with results of a previous study (14), we found that FI-RSV appeared to induce memory T cells with a predominant Th2-like cytokine profile whereas live RSV induced memory T cells with a predominant Th1-like cytokine profile. The actual profiles found were, however, somewhat different. Relative to the results with live-RSV-immunized mice, Graham et al. (14) found an increase in IL-4 mRNA levels and a decrease in IFN-γ mRNA levels after challenge of FI-RSV-immunized mice. In contrast, we found no clear differences in IFN-γ mRNA levels between mice immunized with FI-RSV or live RSV and a less clear difference in IL-4 mRNA. These differences could be methodological, since we used a lower dose of challenge virus, had less virus replication in vaccinated mice, and used BAL specimens instead of whole-lung extracts as the source of mRNA. Time also was a factor in some of the discrepant results. Graham et al. (14) found elevated IL-2 mRNA levels in live-RSV-immunized mice only at 12 h postinfection (a time point we did not study), and we found IL-2 mRNA in the FI-RSV-immunized animals on day 8 (a time point they did not study).

Our results for IL-5, IL-10, IL-12, and IL-13 mRNAs gave new and expanded information on the possible role of cytokines in the pathogenesis of enhanced inflammation in mice. IL-5 and IL-13, both defined as Th2-type cytokines, gave the most distinctive differences between live RSV and FI-RSV immunization. High mRNA levels were found only in FI-RSV-immunized mice. The significance of IL-5 mRNA in these mice is particularly intriguing, since it correlated with the presence of eosinophils in the lung. IL-5 is an eosinophil differentiation factor and is produced primarily by activated $CD4^+$ cells but also by mast cells and eosinophils themselves (9, 42).

IL-10 and IL-12 gave more subtle differences between the two types of immunization. IL-10 mRNA was present after both on day 4, but its level was somewhat decreased in the live-virus-immunized mice on day 8, consistent with a shift toward a Th1-like pattern. IL-12 mRNA, known to be produced by macrophages and B cells (38), was present in all groups but decreased on day 4 in FI-RSV-immunized mice, consistent with a shift toward a Th2-like pattern (Fig. 2B). The lower levels of IL-12 suggest an altered macrophage function in the FI-RSV-immunized animals that could result in reduced NK cell and $CD8^+$ cytotoxic T-lymphocyte activity (22, 38). Since IL-12 can counterbalance the effect of IL-4 and IL-10, lower levels could also increase the effect of these cytokines. On the basis of neutralization studies, both IL-4 and IL-10 are likely to be important to the pathogenesis of enhanced inflammation after FI-RSV immunization in the BALB/c mouse (7, 35).

The goal of these studies of enhanced inflammation in rodents is to understand which components of the FI-RSV cause enhanced inflammation and disease and how to avoid similar effects in future vaccines. Similar studies in mice have not been done for other viruses, but other FI viral vaccines have been used in humans. Atypical measles virus pneumonia was observed in children vaccinated with FI-virus and exposed to natural measles a few years later (5), but no enhanced disease has been reported in association with FI-influenza virus (10, 16) or FI-PIV3 (6, 13) vaccination, suggesting that formalin inactivation alone is not sufficient to cause enhanced disease. Presumably, characteristics of the virus or the infection it causes also contribute to directing the immune response toward enhanced disease. Some groups have suggested that the presence of cellular proteins in FI-RSV may contribute to enhanced inflammation or disease (31, 39). Our data and those from other groups, however, suggest that such proteins are not the key to enhanced inflammation (7, 14). We found similar average responses to live RSV inoculation in mock-immunized and nonimmunized mice and no inflammatory response in FI-RSV-immunized mice challenged with cell control material. Data from Graham et al. (14) suggest that inactivation of RSV may be a factor. They found that killed virus with or without formalin inactivation and with or without alum adjuvant gave the same Th2-like response as FI-RSV administered i.m. or i.n., while live RSV administered i.n. or i.m. induced a Th1-like response. They also noted that a single dose of affinity-purified F protein induced a Th2-like response (14). These observations suggest that subunit or inactivated vaccines may have to be given with adjuvants that direct the immune response toward that associated with live-RSV infection to minimize the chance of enhanced disease. A variety of adjuvants that can be used to modulate the immune response, including cytokines like IL-12 that can stimulate Th1-type responses, have been developed (36, 38). Another approach that we are investigating in BALB/c mice is to prime them with a live RSV vaccine to establish a safe pattern of immunity and boost them with killed virus or subunit vaccine to augment the protective immune response. This approach is supported by the lack of enhanced disease in children older than 2 years, who presumably had had natural infection before FI-RSV vaccination (19, 20).

The results obtained with our control mice provide some information about the inflammatory process in primary RSV infection that can be compared with data from previous studies. Similar to other studies, we found the total number of cells recovered by BAL 4 and 8 days postinfection and a decrease in the $CD4^+/CD8^+$ lymphocyte ratio later in the course of infection (22, 27, 28). Previous studies have shown that IFN-γ, IL-2, IL-6, and tumor necrosis factor alpha are upregulated in response to RSV infection; IL-2 and IL-6 mRNA levels were increased early in the infection, being higher 12 h than 4 days postinfection (14), whereas secreted IL-6 and tumor necrosis factor alpha levels peaked on day 1 but remained detectable for at least 7 days (17). We found little IL-2, IL-4, or IL-5 mRNA on either day 4 or 8; IFN-γ, IL-10, and IL-12 mRNAs were present on both day 4 and day 8 but had increased by day 8. Taken together, these studies show that the local cytokine response to primary RSV infection is of mixed type but is mainly Th1-like.

We chose to use PCR to measure cytokine mRNA expression to maximize sensitivity; in so doing, we were left with the problems of quantification by PCR. By using highly sensitive PCR assays, we sacrificed some of our ability to distinguish differences at high template concentrations. The variability in the efficiency of PCR further confounds the detection of differences in amounts of mRNA. Use of an internal PCR standard theoretically could eliminate some of this variability (40). We chose not to normalize our results to the amount of an internal housekeeping gene, such as β-actin, or the number of cells. Normalization to the number of cells did not affect our results qualitatively. However, we did screen for adequate quality and quantity of mRNA cDNA by reviewing the inten-

sity of the PCR band for β-actin mRNA. Furthermore, normalizing to the cell number or the amount of a housekeeping gene mRNA does not account for differences in cell types that might contribute to differences in cytokine mRNA. Thus, differences in cytokine mRNA levels may reflect differences in both number of cells, e.g., $CD4^+$ and $CD8^+$ T cells, and types of cells, e.g., Th1 and Th2 memory T cells, or even the presence of other cells, e.g., eosinophils in BAL fluid from FI-RSV-immunized mice, which could be a source of IL-5 mRNA. Despite these limitations, the consistency in the patterns of cytokine mRNA expression between mice in the same treatment group and the large differences in relative amounts of mRNA for key cytokines support the validity of our findings. The role that differences in cytokine expression play in the disease process, however, will have to be determined in future studies.

In conclusion, we have identified several measures of the inflammatory response in BAL specimens that clearly distinguish FI-RSV from live-virus immunization in BALB/c mice. These differences include the number of cells, the ratio of $CD4^+$ to $CD8^+$ cells, the presence of granular cells (by flow cytometry analysis) and eosinophils (by light microscopy), and the pattern of cytokine mRNA. The observations that live-virus infection in humans induces memory T cells with similar cytokine patterns to those in mice (3) and that pulmonary eosinophilia was noted in the two FI-RSV-immunized children who died of natural RSV infection (20) suggest that the response to RSV in the mouse parallels the response in humans. The mouse model as outlined in this study should provide a reasonable initial screen for disease-enhancing potential of candidate RSV vaccines should also provide a means to clarify the pathogenesis of enhanced inflammation in mice and probably of enhanced disease in humans.

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